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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
High levels of *Wilms' Tumor 1 (WT1)* mRNA in breast tumors have been linked with poor prognosis for breast cancer patients. However, the function of WT1 protein in breast cancer was not known. We reported the expression of WT1 protein in 9 out of 10 human breast cancer cell lines. We observed that the levels of WT1 protein correlated with the proliferation of breast cancer cells. When the proliferation of breast cancer cells was stimulated by 17β-estradiol, WT1 protein expression increased. But when the proliferation of breast cancer cells was inhibited by tamoxifen or all-*trans* retinoic acid, WT1 protein expression decreased. To determine whether WT1 regulates breast cancer cell proliferation, antisense oligodeoxynucleotides specific for the translation initiation site of the *WT1* mRNA were used to inhibit the expression of WT1 protein. We demonstrated that WT1 protein is vital to the proliferation of breast cancer cells since downregulation of WT1 protein expression led to breast cancer growth inhibition. WT1 protein may be used as a novel therapeutic target in the fight against breast cancer.

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INTRODUCTION

The Wilms' Tumor 1 (WT1) protein and mRNA is expressed in human breast tumors and breast cancer cell lines [1-4]. High levels of WT1 mRNA have been correlated with poor prognosis for breast cancer patients [3]. We observed that the expression of WT1 protein was correlated with breast cancer cell proliferation. We hypothesize that WT1 protein contributes to breast tumor progression by deregulating cell proliferation and apoptosis. Deregulation of proliferation and survival pathways has been associated with chemoresistance in many tumors. Therefore, we also hypothesize that WT1 regulates chemoresistance in breast cancer cells. WT1 mRNA undergoes two independent splicing events that result in four different isoforms, which can bind to different DNA promoter elements and different protein partners, are expressed in breast tumors and breast cancer cell lines. In this proposal, we will determine the mechanisms and the isoforms by which WT1 deregulates breast cancer cell proliferation and apoptosis.

BODY

In our Statement of Work, Task 1 is to determine whether WT1 overexpression increases the proliferation and survival of breast cancer cells in cell culture models.

We have transfected MCF-7 breast cancer cells with the "A" and the "D" isoforms of the WT1 gene. Stable transfectant clones were selected using the G418 antibiotic, and WT1 overexpression was confirmed by Western blot and reverse transcription-polymerase chain reaction (RT-PCR). Flow cytometry, cell counting, and the CellTiter 96 Aqueous nonradioactive proliferative assays suggest that the "D" isoform increases proliferation while the "A" isoform suppresses proliferation. However, before we could confirm these observations, the expression of WT1 protein in the stable transfectants unexpectedly decreased

to basal levels. We are now doing another round of stable transfection. But this time, the transfection is carried out in MCF-7 as well as MDA-MB-435 breast cancer cells. Furthermore, the plasmids now have an additional FLAG-tag. If WT1 overexpression could not be maintained in breast cancer cell lines, we will then have to make new WT1 gene plasmids that are regulated by inducible promoters (such as the tet-on or the tet-off system). These unexpected issues have set us back somewhat, but we still expect to complete the goals of our proposal within the original 3-year plan.

KEY RESEARCH ACCOMPLISHMENTS

We demonstrated that WT1 protein expression is regulated by estrogen and anti-estrogen, and that WT1 protein regulates breast cancer cell proliferation [4]. Meanwhile, we have preliminary data indicating that the HER2/*neu* oncogene uses the Akt pathway to increase the expression of WT1 protein to stimulate breast cancer cell proliferation. These results may increase our understanding of how the HER2/*neu* oncogene promotes breast cancer progression.

REPORTABLE OUTCOMES

Zapata-Benavides, P., Tuna, M., Lopez-Berestein, G., and Tari, A. M. Downregulation of Wilms' Tumor 1 protein inhibits breast cancer proliferation. Biochem. Biophys. Res. Commun., *295:*784-790, 2002.

Tuna, M. and Tari, A. M. HER2/neu increases the expression of Wilms' Tumor 1 (WT1) protein via the Akt pathway in breast cancer cells. Abstract was selected as an oral presentation

in the 2003 Annual American Association for Cancer Research Symposium, and Dr. Tuna was selected to receive a prestigious Scholar-in-Training Award.

CONCLUSIONS

We are the first group to demonstrate that WT1 protein regulates the proliferation of breast cancer cells, and that WT1 protein may be used as a novel therapeutic target in the fight against breast cancer.

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Downregulation of Wilms' tumor 1 protein inhibits breast cancer proliferation

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Abstract

High levels of *Wilms' Tumor 1* (*WT1*) mRNA have been correlated with poor prognosis in breast cancer patients. However, the function of WT1 protein in breast cancer is not known. We observed that the levels of WT1 protein correlated with the proliferation of breast cancer cells. When the proliferation of breast cancer cells was stimulated by 17β-estradiol, WT1 protein expression increased. But when the proliferation of breast cancer cells was inhibited by tamoxifen or all-*trans* retinoic acid (ATRA), WT1 protein expression decreased. We hypothesize that WT1 protein plays a role in regulating breast cancer cell proliferation. Using liposome-incorporated WT1 antisense oligodeoxynucleotides, we found that downregulation of WT1 protein expression led to breast cancer growth inhibition and reduced cyclin D1 protein levels. These results indicate that WT1 protein contributes to breast cancer progression by promoting breast cancer cell proliferation.

Keywords: WT1; Breast cancer cell growth; 17β-Estradiol; ATRA; Cyclin D1

The *Wilms' Tumor 1* (*WT1*) gene was originally identified as a gene that is deleted or rearranged in many cases of hereditary Wilms' tumor [1,2], a childhood kidney neoplasm. In addition to germ-like abnormalities, somatic mutations of *WT1* as well as loss of heterozygosity at the 11p13 locus harboring the *WT1* gene have been reported in sporadic Wilms' tumors. Mutations in the *WT1* gene have also been associated with three distinct syndromes in humans, namely WAGR syndrome (Wilms' tumor, aniridia, genitourinary anomalies, and mental retardation), Denys–Drash syndrome, and Frasier syndrome [3]. The *WT1* gene encodes a zinc finger-containing nuclear protein with DNA- and RNA-binding activities [3]. WT1 binds to CG-rich, early growth response consensus sequences, as well as TG-rich sequences [4–6]. Microinjection of WT1 cDNA into quiescent cells blocked serum-induced cell cycle progression [7]. Overexpression of the *WT1* gene inhibited the anchorage-independent growth of *ras*-transformed NIH-3T3 cells [8]. Thus, *WT1* appears to act as a tumor suppressor gene. The tumor-suppressive function of WT1 has been attributed to its ability to suppress the expression of growth-related genes, including insulin-like growth factor-I receptor [9,10], insulin-like growth factor-II [11], platelet-derived growth factor-A [12], transforming growth factor-β [13], c-myc [14], Bcl-2 [14,15], amphiregulin [16], E-cadherin [17], epidermal growth factor receptor [18], and connective tissue growth factor [19].

On the other hand, the *WT1* gene has been correlated with the pathogenesis and progression of leukemia. Higher levels of *WT1* mRNA had been found in leukemia cells than normal hematopoietic cells [20–22]. Leukemia patients who had high levels of *WT1* mRNA had increased drug resistance and worse overall survival than patients who had low levels of *WT1* mRNA [23,24]. *WT1* mRNA expression was downregulated during differentiation of leukemia cells [25,26]. Furthermore, decreased expression of WT1 protein levels led to growth inhibition and apoptosis in leukemia cells in vitro [27,28] and in vivo [29]. Thus, *WT1* appears to have an oncogenic role in leukemia. The WT1 protein

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also appears to have a growth regulatory role in some solid tumor cells, such as gastric cancer, lung cancer, and ovarian cancer cell lines [30].

WT1 protein and mRNA expression had been found in breast tumors [31–33]. Loeb et al. [32] demonstrated that *WT1* mRNA and protein are expressed in nearly 90% of breast cancers, but not in most normal breast tissues. Silberstein et al. [31] suggested that there was an association of WT1 protein expression with a biologically aggressive phenotype of breast cancer. Recently, Miyoshi et al. [33] correlated high levels of *WT1* mRNA with poor prognosis in breast cancer patients. But it is not known how WT1 protein contributes to breast tumorigenesis. We observed that the expression of WT1 protein correlated with the proliferation of human breast cancer cell lines. When breast cancer cells were growth stimulated by 17β-estradiol, WT1 protein levels increased. When breast cancer cells were growth inhibited by tamoxifen or all-*trans* retinoic acid (ATRA), WT1 protein levels decreased. Here, we determine whether WT1 protein plays a role in regulating breast cancer cell proliferation.

Materials and methods

Cell culture. Human breast cancer cell lines: MCF-7, BT-474, T-47D, MDA-MB-361, Hs578T, SKBr-3, MDA-MB-453, MDA-MB-231, MDA-MB-468, and MDA-MB-435 were obtained from the American Type Culture Collection (Manassas, VA). They were propagated in Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with 10% fetal bovine serum (FBS). K562 cells were obtained from Dr. Ralph Arlinghaus (The University of Texas M.D. Anderson Cancer Center) whereas HeLa cells were obtained from Dr. Tetsuo Ashizawa (Baylor College of Medicine). K562 and HeLa cells were used as positive and negative controls, respectively. The control cells were propagated in RPMI 1640 medium supplemented with 10% FBS. All cell lines were incubated in 95% air and 5% CO_2 at 37 °C.

Ligands and antibodies. 17β-Estradiol, tamoxifen, ATRA, and anti-β-actin monoclonal antibody were purchased from Sigma Chemical (St Louis, MO). Monoclonal antibodies specific for WT-1 (6F-H12), cyclin D1, and Grb2 were obtained from DAKO (Carpinteria, CA), Neomarkers (Fremont, CA), and BD Transduction Laboratories (San Diego, CA), respectively. Anti-mouse antibodies conjugated with horseradish peroxidase were purchased from Amersham Life Sciences (Cleveland, OH).

Incubation of MCF-7 cells with 17β-estradiol and/or tamoxifen. MCF-7 cells were seeded in triplicate in six-well plates (1.5×10^5 cells per well) in DMEM/F12 medium supplemented with 10% FBS. After 24 h, the medium was removed, and cells were washed three times with phosphate-buffered saline (PBS). Cells were then cultured in phenol red-free DMEM/F12 medium supplemented with 10% dextran charcoal stripped serum (DCS) and 10 nM 17β-estradiol. For tamoxifen treatment, cells were incubated with 10 nM 17β-estradiol and various concentrations of tamoxifen (0, 0.1, 1, or 5 μM).

Incubation of T-47D cells with retinoids. T-47D cells were plated at 0.5×10^5 cells/well in six-well plates in 2 mL of DMEM/F12 medium supplemented with 10% FBS. After overnight adherence, ATRA was added to cells at a final concentration of 2.5 μM in subdued light. After 5 days of incubation, cells were harvested, counted, and assayed for WT1 protein expression.

Western blots. Breast cancer cells were harvested and lysed. Protein concentration was determined by using the Bio-Rad DC reagent kit. Proteins were electrophoresed on 12% SDS–polyacrylamide gels and transferred to nitrocellulose membranes. Protein bands were visualized by enhanced chemiluminescence (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Intensities of protein bands were quantified by densitometry using an Alpha Imager application program (Alpha Innotech, San Leandro, CA).

Liposome-incorporated WT1 antisense oligonucleotides and cell treatment. P-ethoxy oligodeoxynucleotides (oligos), purchased from Oligos Etc. (Wilsonville, OR), were incorporated into dioleoylphosphatiylcholine (DOPC) liposomes as previously described [34]. The sequence of the WT1 antisense oligos targeted against the translation initiation site is 5′-GTC GGA GCC CAT TTG CTG-3′ and the sequence of the control oligos is 5′-GGG CTT TTG AAC TCT GCT-3′ [27,28]. Breast cancer and leukemia cells were plated in 96-well plates (2×10^3 cells per well) in DMEM/F12 supplemented with 10% FBS and allowed to adhere overnight. Then, various concentrations (0, 3, 6, or 12 μM) of liposomal WT1 antisense (L-WT1) or liposomal control (L-control) oligos were added to the cells and incubated for 72 h. Cell growth was determined by using the CellTiter 96 Aqueous nonradioactive proliferation assay (Promega, Madison, WI).

Light microscopic evaluation of cell growth. MCF-7 and MDA-MB-453 cells were seeded in six-well plates (1.0×10^5 cells per well) in DMEM/F12 medium supplemented with 10% FBS. After 24 h, the cells were treated with 12 μM L-WT1 or L-control oligos for 3 days, examined under light microscopy at 100× magnification, and photographed with Kodak gold 400 film.

Results

Expression of WT1 protein in human breast cancer cell lines

The expression levels for WT1 protein were determined in human breast cancer cell lines: MCF-7, BT-474, T-47D, MDA-MB-361, Hs578T, SKBr3, MDA-MB-453, MDA-MB-231, MDA-MB-468, and MDA-MB-435. As reported in the literature [35], WT1 was expressed at high levels as two protein bands, 52 and 54 kDa, in K562 cells whereas WT1 protein was essentially undetectable in HeLa cells (Fig. 1). WT1 protein expression was detected in all human breast cancer cell lines (Fig. 1). However, the expression of WT1 protein was heterogeneous among the breast cancer cell lines. T-47D cells expressed the highest levels of WT1 protein, whereas MDA-MB-231 and MDA-MB-361 cells expressed very low levels of WT1 protein (Fig. 1). Both the 52 and 54 kDa WT1 protein bands were seen in T-47D and MDA-MB-468 cells, but only the upper 54 kDa WT1 protein band was seen in the remaining breast cancer cell lines (Fig. 1).

WT1 protein expression is stimulated by 17β-estradiol but inhibited by tamoxifen

17β-Estradiol is known to stimulate various gene expressions. We determined whether 17β-estradiol modulates WT1 protein expression. When MCF-7 cells were incubated with 10 nM 17β-estradiol in phenol red-free DMEM/F12 medium supplemented with 10% DCS

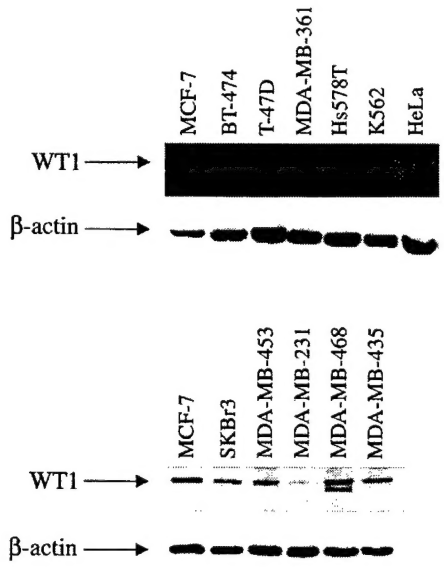

Fig. 1. Expression of WT1 protein in human breast cancer cell lines. WT1 protein expression was analyzed by Western blot analysis. Fifty μg cell lysates were loaded onto 12% SDS–PAGE. WT1 protein expression was determined by monoclonal anti-WT1 antibodies. K562 and HeLa cells were used as positive and negative controls, respectively.

for 3 days, a 100% increase in cell number was observed as compared with cells incubated in DMEM/F12 medium supplemented with 10% DCS alone (Fig. 2A). High expression of WT1 protein was found in MCF-7 cells cultured in 10% FBS (Fig. 2B). But when MCF-7 cells were cultured in phenol red-free DMEM/F12 medium supplemented with 10% DCS, the expression of WT1 protein decreased by about 60% (Fig. 2B). Incubation of MCF-7 cells with 10 nM 17β-estradiol could increase the expression of WT1 protein to a level similar to that seen in 10% FBS (Fig. 2B).

To determine whether the effects of 17β-estradiol on the expression levels of WT1 were specific, MCF-7 cells were treated with the estrogen antagonist tamoxifen. MCF-7 cells were incubated with 10 nM of 17β-estradiol and different concentrations (0.1, 1, and 5 μM) of tamoxifen for 3 days. Tamoxifen decreased the growth stimulatory effects of 17β-estradiol in a dose-dependent manner (Fig. 2A). In wells treated with 10 nM 17β-estradiol plus 1 and 5 μM tamoxifen, cell numbers were lower (12% and 49%, respectively) than cells treated with 17β-estradiol only (Fig. 2A). Tamoxifen also decreased the WT1 stimulatory effects by 17β-estradiol in a dose-dependent manner (Fig. 2B). In wells treated with

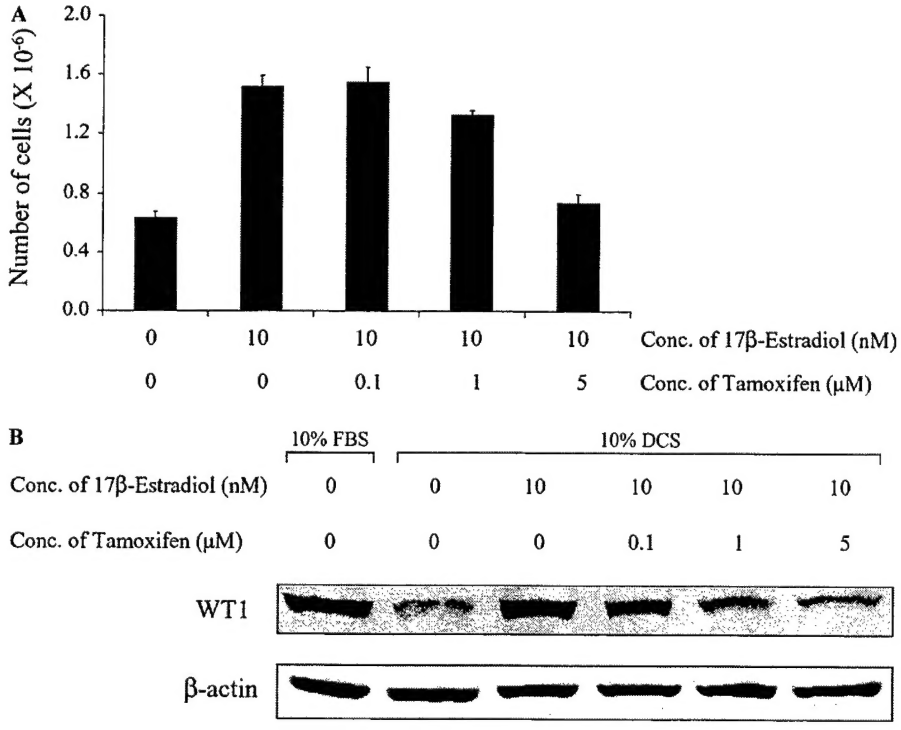

Fig. 2. 17β-Estradiol increased WT1 protein expression but tamoxifen decreased WT1 expression in MCF-7 cells. (A) MCF-7 cells were seeded in triplicate (at 1.5×10^5 per well) in six-well plates in DMEM/F12 medium supplemented with 10% FBS and incubated for 24 h. The medium was then changed to phenol red-free DMEM/F12 medium supplemented with 10% DCS and 10 nM 17β-estradiol. Various concentrations of tamoxifen (0, 0.1, 1, or 5 μM) were added to cells and the cells were then incubated for an additional 72 h. Cells were harvested and counted by trypan blue dye exclusion. The numbers of MCF-7 cells are expressed as means ± SD. (B) Fifty μg proteins were used to analyze WT1 expression by Western blot analysis. Data were quantified by scanning densitometry and WT1 levels were normalized to β-actin levels.

10 nM 17β-estradiol plus 1 and 5 μM tamoxifen, WT1 expression decreased by 32% and 51%, respectively (Fig. 2B). These data indicate that 17β-estradiol can stimulate the expression of WT1 protein in breast cancer cells and that the expression levels of WT1 protein correlated with the growth of MCF-7 cells as stimulated by 17β-estradiol.

WT1 protein expression is suppressed by ATRA in breast cancer cells

It has been shown that ATRA reduces WT1 levels in HL60 leukemia and F9 tetracarcinoma cells [25,36]. Here, we determined whether ATRA could also reduce WT1 expression in breast cancer cells. As a positive control [37], we confirmed that ATRA decreased the levels of the cyclin D1 protein (Fig. 3). Under these conditions, ATRA was found to inhibit WT1 protein expression by 31% (Fig. 3). The inhibition was predominantly in the upper 54 kDa WT1 protein band. ATRA-mediated suppression of WT1 protein levels was correlated with ATRA-mediated growth inhibition, as T-47D cells incubated with 2.5 μM ATRA for 5 days had 69% lower cell numbers than untreated cells (data not shown).

Downregulation of WT1 protein expression leads to growth inhibition in breast cancer cells

Since we observed that the levels of WT1 protein correlated with the proliferation of breast cancer cells, we hypothesized that WT1 protein plays a role in regulating breast cancer cell proliferation. L-WT1 oligos were used to downregulate WT1 protein expression and effects of WT1 downregulation on breast cancer cell proliferation were determined.

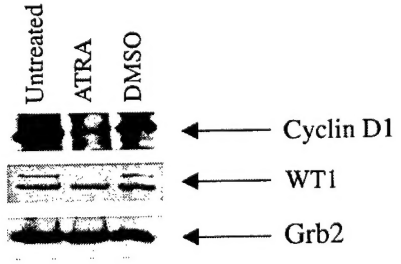

Fig. 3. ATRA suppressed WT1 levels in T-47D cells. T-47D cells were seeded in triplicate (at 0.5×10^5 cells per well) in six-well plates in DMEM/F12 medium supplemented with 10% FBS. ATRA was added to cells at a final concentration of 2.5 μM. DMSO, added at a final concentration of 0.025%, was used as a negative control. After 5 days of incubation, cells were harvested and counted. Twenty-five μg proteins were used to assay for WT1 and cyclin D1 expression. ATRA is known to inhibit cyclin D1 levels in T-47D cells and was used here as a positive control. Data were quantified by scanning densitometry and WT1 levels were normalized to Grb2 levels.

We first confirmed that L-WT1 oligos could inhibit the growth of K562 leukemia cells [27,28] in a dose-dependent manner (Fig. 4A). We then determined the effects of L-WT1 oligos on the proliferation of breast cancer cells. L-WT1 oligos, but not L-control oligos, induced dose-dependent growth inhibition in MCF-7 and MDA-MB-453 cells (Fig. 4B). Maximal growth inhibition (>90%) was observed at 12 μM concentration of L-WT1 oligos; therefore, this concentration was used for the subsequent experiments. We expanded our

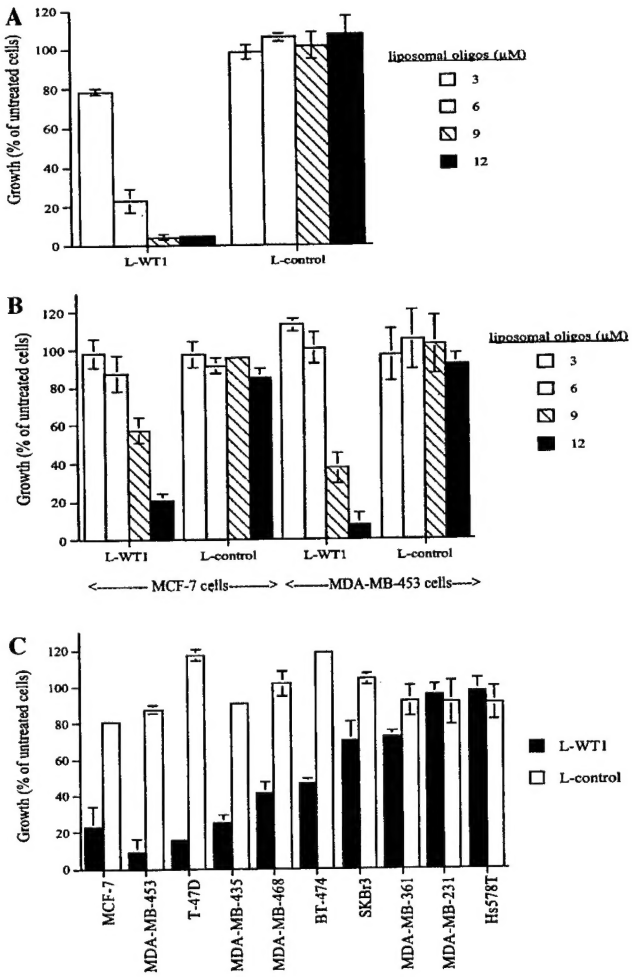

Fig. 4. L-WT1 selectively inhibited the growth of breast cancer cell lines. (A) K562 leukemia cells were used as a positive control and incubated with increasing concentrations (0, 3, 6, 9, and 12 μM) of L-WT1 and L-control oligos for 3 days. Cell growth was determined by the CellTiter 96 Aqueous nonradioactive proliferation assay. The growth of treated cells is expressed as percentage of untreated cells ± SD. (B) MCF-7 and MDA-MB-453 cells were incubated with 0–12 μM L-WT1 and L-control oligos. After 3 days of incubation, cell growth was determined by the CellTiter 96 Aqueous nonradioactive proliferation assay. The growth of treated cells is expressed as percentage of untreated cells ± SD. (C) Breast cancer cell lines were incubated with 12 μM L-WT1 oligos (dark bars) or L-control oligos (light bars) for 3 days. Cell growth was determined by the CellTiter 96 Aqueous nonradioactive proliferation assay. The growth of treated cells is expressed as percentage of untreated cells ± SD.

findings to the other breast cancer cell lines. L-WT1 oligos inhibited the growth of 8 out of 10 breast cancer cell lines, with greater than 50% effects in MCF-7, MDA-MB-453, T-47D, and MDA-MB-435 cells (Fig. 4C). Under the same conditions, approximately 50% growth inhibition was observed in MDA-MB-468 and BT-474 cells while less than 50% growth inhibition was observed in SKBr-3 and MDA-MB-361 cells (Fig. 4C). No growth inhibition was observed in MDA-MB-231 and Hs578T cells (Fig. 4C).

Western blot analysis was performed to ensure that L-WT1, but not L-control, oligos could specifically inhibit WT1 protein expression. L-WT1 oligos selectively inhibited WT1 protein expression (~46% lower) in MDA-MB-453 cells (Fig. 5). Furthermore, a decrease in cyclin D1 protein expression (~40% lower) was observed when MDA-MB-453 cells were treated with L-WT1 oligos (Fig. 5). As expected, WT1 protein expression was not affected by L-control oligos (Fig. 5).

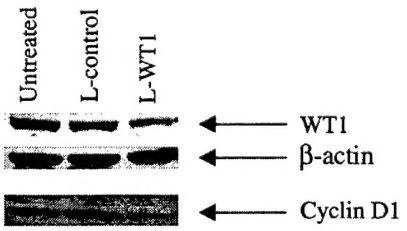

Fig. 5. L-WT1 oligos decreased WT1 protein expression in MDA-MB-453 cells. MDA-MB-453 breast cancer cells were incubated with 12 μM L-WT1 or L-control oligos for 3 days. Cells were harvested and 50 μg protein lysates were loaded on 12% SDS–PAGE. The levels of WT1 and cyclin D1 protein were determined by Western blot analysis. Data were quantified by scanning densitometry and WT1 and cyclin D1 levels were normalized to β-actin levels.

Using light microscopy, we observed that L-WT1 oligos reduced the number of MCF-7 and MDA-MB-453 cells as compared with untreated cells (Fig. 6). But L-control oligos did not decrease the number of MCF-7 and MDA-MB-453 cells (Fig. 6).

Discussion

Our data demonstrate that WT1 protein regulates breast cancer cell proliferation, since downregulation of WT1 protein led to growth inhibition in breast cancer cell lines. Our results may explain the findings of Miyoshi et al. [33] who reported that high levels of *WT1* mRNA predict poor prognosis in breast cancer patients. WT1 protein is vital to the proliferation of breast cancer cells, regardless of whether the cells are estrogen receptor (ER)-positive or -negative. We did not find any correlation between the basal expression of WT1 protein in breast cancer cells and their response to inhibition by L-WT1 oligos. We could also not find a correlation between the status of the p53 protein expressed in breast cancer cells and their response to inhibition by L-WT1 oligos, since MCF-7 is the only breast cancer cell line that expresses the wild-type p53 protein [38]. However, one possible mechanism by which WT1 protein contributes to breast cancer proliferation is by regulating the levels of the cyclin D1 protein, which is known to be important to breast cancer progression [39]. This is because downregulation of WT1 protein led to reduction in cyclin D1 protein levels. The *cyclin D1* promoter contains CG-rich elements that resemble WT1 response elements [40]. We are currently investigating whether WT1 regulates cyclin D1 expression at the transcriptional level.

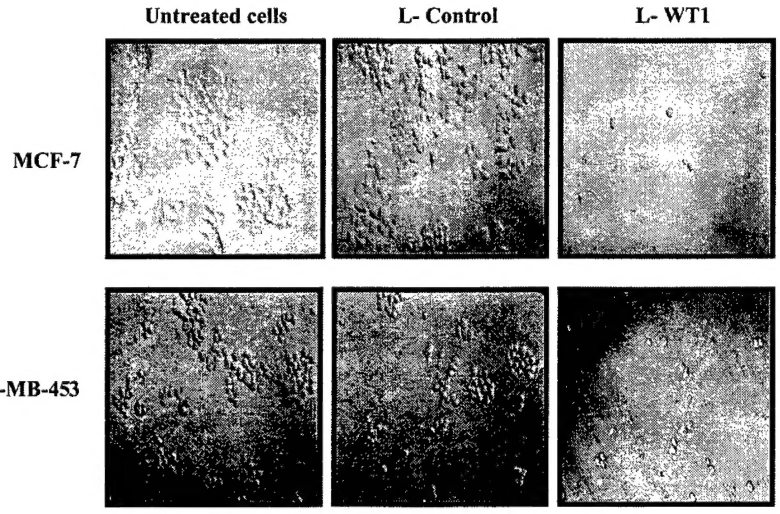

Fig. 6. Reduction in numbers of breast cancer cells by L-WT1 oligos. MCF-7 and MDA-MB-453 cells were treated with 12 μM L-WT1 or L-control oligos for 3 days and observed under light microscopy.

Our data also demonstrate that steroid hormones, 17β-estradiol, and ATRA regulate WT1 protein expression in breast cancer cells. 17β-Estradiol is a positive regulator of WT1 expression, whereas ATRA is a negative regulator of WT1. 17β-Estradiol and ATRA mainly induce their biological effects in ER-positive, but not ER-negative, breast cancer cells. However, the mechanisms by which these ligands regulate WT1 expression are not known. The promoter of the *WT1* gene does not contain any ER or retinoic acid receptor (RAR) response element [41]. Thus, it is unlikely that WT1 expression is mediated directly by ER or RARs. Since WT1 protein is vital to the proliferation of ER-negative breast cancer cells, it will also be interesting to determine what growth factors and/or receptors regulate WT1 expression in ER-negative breast cancer cells.

High levels of methylation had been found in the promoter of the *WT1* gene in breast cancer cells. Nonetheless, *WT1* mRNA and protein are expressed in breast tumors and breast cancer cell lines [32]. Here, we demonstrate that WT1 protein is vital to the proliferation of breast cancer cells. Our data support earlier observations that WT1 may play a role in the pathogenesis of breast cancer [31,33]. WT1 protein may therefore be used as a novel therapeutic target in the fight against breast cancer.

Acknowledgments

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